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Endopeptidase-24.11 in pig lymph nodes

Purification and immunocytochemical localization in reticular cells

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Endopeptidase-24.11 (EC 3.4.24.11), a widely distributed cell-surface endopeptidase in pig tissues, was purified by immunoaffinity chromatography from its second most abundant source, lymph nodes. The detergent-solubilized enzyme is a glycoprotein with an apparent subunit M_r of 91000, by electrophoresis in the presence of SDS. This value is intermediate between those observed in preparations from kidney and intestine. The specific activity (^{125}I-labelled insulin B-chain as substrate) was similar to that prepared from other sources. Immuno-peroxidase and immunofluorescent cytochemical methods with either a monoclonal antibody, GK7C2, or an affinity-purified polyclonal antiserum, RP109, were used to establish the distribution and localization of the antigen in lymph nodes. Examination of many nodes confirmed the variability of endopeptidase-24.11 content from node to node. Pig lymph nodes are composed of functionally discrete nodelets and are anatomically inverted, with medulla being located peripheral to the cortex. Endopeptidase-24.11 was present in medulla, paracortex and cortex. The medulla, containing relatively few lymphocytes, stained more intensely than other zones. Lymphocyte-rich areas stained only weakly, but antigen was detectable in the centres of follicles and more strongly in a band surrounding them. The pattern of staining was reticular in appearance in all zones. In primary cell cultures, set up after enzymic disruption of nodes, the immuno-positive cells were found to be adherent to glass or plastic and to exhibit a fibroblastic morphology. Diffuse surface immunofluorescence and brighter intracellular immunofluorescence in granules were observed in these cells in the first few days of culture, but by the fourth day no immuno-positive cells remained and the fibroblasts that grew to confluence were somewhat different in morphology. The cells expressing the endopeptidase-24.11 antigen did not express Ia antigen and were clearly distinct from antigen-presenting dendritic cells. In appearance and properties they belong to the group described as reticular cells.

INTRODUCTION

From its distribution, topology and specificity, endopeptidase-24.11 (EC 3.4.24.11) has the potential to play an important role in the inactivation of peptides at various cell surfaces. In kidney it is one of the major constituents of the microvillar membrane, and is readily purified from this organ (Kerr & Kenny, 1974; Fulcher & Kenny, 1983). Like other microvillar hydrolases it is an ectoenzyme, with the active site exposed at the external surface of the membrane (Kenny & Maroux, 1982; Kenny *et al.*, 1983). The enzyme is also present in brain, where it is responsible for the so-called 'enkephalinase' activity (Fulcher *et al.*, 1982; Matsas *et al.*, 1983), and from which source it has also been purified (Relton *et al.*, 1983). Although susceptible peptides are hydrolysed at predictable bonds (those involving the amino groups of hydrophobic amino acid residues), the efficiency of hydrolysis of different peptides varies over a wide range of values, with tachykinins, including substance P, being among the best substrates (Matsas *et al.*, 1984*a*,*b*; Hooper *et al.*, 1985). In brain synaptic membrane preparations, endopeptidase-24.11 appears to be the major enzyme responsible for inactivating certain peptide neurotransmitters. However, relative to kidney microvillar membranes, synaptic membranes exhibit rather less than 1% of the activity, assessed either enzymically or by an i.r.m.a. (Matsas *et al.*, 1985). In contrast, the i.r.m.a. has shown that lymph nodes are the second richest source of endopeptidase-24.11 (Gee *et al.*, 1985), and, in view of their overall mass, lymph nodes may well contain the bulk of the enzyme in the body. The i.r.m.a. values revealed great variation from node to node, and showed much less of the antigen in other lymphoid organs such as spleen and thymus. These observations suggested that the abundance of the endopeptidase might be related to the immune response. In the present paper we report the isolation of the enzyme from pig lymph nodes, and show, by immunocytochemistry, that it has a characteristic pattern of distribution in lymph nodes, typical of reticular cells, a conclusion which is supported by studies on primary cultures of lymph-node cells.

EXPERIMENTAL

Materials

Piglets were obtained from the University of Leeds Field Station. Tissues from slaughterhouse pigs were kindly donated by Asda Farm Products, Lofthousegate, W. Yorks, U.K.

Abbreviation used: i.r.m.a., immunoradiometric assay.

Purification of endopeptidase-24.11 from lymph nodes

Mesenteric lymph nodes (200 g) were obtained from the slaughterhouse, and dissected free of fat and connective tissue. The nodes were homogenized, in a blender, in 850 ml of 150 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.4. After removal of the 580 *g* × 60 min pellet, the supernatant was centrifuged at 17500 *g* for 2 h and the pellet resuspended in 600 ml of 5 mM-Tris/HCl buffer, pH 7.4. Triton X-100 was added to give a detergent/protein ratio of 7:1 (w/w). After standing for 1 h at 20 °C, the suspension was centrifuged at 17500 *g* and the supernatant loaded on a column (50 mm diam. × 50 mm) of DEAE-cellulose equilibrated in the same buffer. The column was washed (10 column volumes), a linear gradient of NaCl (0–200 mM) in the same buffer was applied and the active fractions were pooled. Subsequent purification used a monoclonal antibody (GK7C2) affinity column as previously described (Gee *et al.*, 1983). All buffers contained 0.1% Triton X-100.

Medium

The culture medium (referred to as 'medium' in the text below) was Dutch modified RPMI 1640 (Flow Laboratories, Cat. no. 12-609-54) containing 1 mM-pyruvate, 4 mM-glutamine, penicillin (50 units/ml), streptomycin (50 μg/ml) and 10% (v/v) foetal-calf serum.

Isolation of lymph-node cells

Lymph nodes were removed from slaughterhouse pigs as aseptically as possible. A cell suspension was prepared from 1–2 g of lymph nodes by incubating small tissue blocks (about 5 mm × 5 mm × 5 mm cubes) in 10 ml of serum-free medium containing Dispase (Boehringer–Mannheim) (200 μg/ml), deoxyribonuclease I (Sigma Chemical Co., code D0876) (100 μg/ml) and collagenase (Sigma type IV, code C5138) (10 μg/ml). After 1 h of incubation at 37 °C rotating on a Spiramix (Denley Scientific Co.), the tissue blocks were gently teased apart with needles, and the resulting suspension was decanted free of the remaining stroma. The suspension was left to stand for 5 min to remove large aggregates, and the supernatant was carefully removed from above the sediment. After being washed twice with medium (by centrifugation at 100 *g* for 10 min), the cells were cultured at 5×10^6 cells/ml in medium containing 10% foetal-calf serum, either on coverslips in six-well culture plates or in Lab-Tek chamber/slides (Miles Laboratories, Code no. 46.481.8).

Immunocytochemical methods

Lymph nodes were obtained from piglets (2–8 weeks old) of various strains, killed by exsanguination under anaesthesia with Small Animal Immobilon (Reckitt & Colman) (0.1 ml/kg). Tissues were quickly removed and cooled on ice. Blocks were prepared, frozen in isopentane cooled in liquid N_2 and stored in liquid N_2. These are referred to as 'fresh blocks'.

For perfusion-fixation, the piglets were premedicated with Immobilon and were anaesthetized with halothane, the chest was opened and a polythene cannula inserted into the left ventricle. Perfusion, from a reservoir 1.2 m above the table, was started (coincidently with an incision of the right atrium) with 400 ml of 150 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.4, containing 20 mg of heparin (Sigma, code H0880). Fixation was achieved by continuing perfusion with 2 litres of 4% (w/v) paraformaldehyde in the same buffer. The flow rate throughout the procedure was approx. 80 ml/min. Tissues were then removed, left in the same fixative for a further 2 h, and blocks were prepared and frozen as described above.

Sections (5–10 μm) were cut at −15 °C on a Leitz Kryostat, taken up on coverslips, air-dried, and were fixed by immersion for 10 min in 4% paraformaldehyde in buffered saline (Gee *et al.*, 1983), or, occasionally, fresh blocks were fixed by immersion in acetone at 20 °C for 5 min.

Cultured cells were prepared for immuno-staining in one of two ways. Those that had adhered to glass or to Miles chamber/slides were gently washed free of non-adherent cells with a Pasteur pipette, and were fixed with 4% paraformaldehyde. Cells that did not adhere were attached as follows. Glass coverslips (22 mm × 22 mm) were coated with poly-L-lysine (Sigma type 1B, code P1886) (50 μg/ml for 1 h at 20 °C). The coverslips were placed in six-well culture plates, and overlaid with 2 ml of serum-free RPMI medium. The plates were centrifuged at 200 *g* for 10 min (MSE Coolspin centrifuge), and allowed to stand for a further 10 min at room temperature to assist in the attachment of the cells. After this time the overlying medium was removed, and 4% paraformaldehyde, in buffered saline, was introduced gently into each well. The fixed sections or cells were washed in 150 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.4 (Tris–saline), and treated for 20 min with 0.2% gelatin in Tris–saline. For immunofluorescence, the samples were treated to reduce endogenous fluorescence with 1% $NaBH_4$ in Tris–saline for 5 min. GK7C2 monoclonal antibody (hybridoma culture medium) or a rabbit polyclonal antibody RP109, raised to kidney endopeptidase-24.11 (1 μg/ml in gelatin/Tris–saline), were the primary antibodies. RP109 antibody had been affinity-purified on a column of CNBr-activated Sepharose 4B to which the endopeptidase had been coupled, and eluted with 0.2 M-glycine/HCl buffer, pH 2.3. The first antibody was incubated at 20 °C with the sample for 2 h. The second antibodies were affinity-purified fluorescein isothiocyanate conjugates of sheep anti-(mouse IgG) antibody [$F(ab')_2$ fragment] (Sigma, code F2883) or goat anti-(rabbit IgG) antibody (Sigma, code F0382). They were diluted (1:100) in gelatin/Tris–saline containing 1 mg of an IgG fraction from pig serum/ml, and incubated with the samples at 20 °C for 1 h. For immuno-peroxidase cytochemistry, endogenous peroxidase activity was quenched by treatment with 0.01% phenylhydrazine in Tris–saline at 37 °C for 30 min. The first antibodies were as described above. The second antibodies were either biotinylated sheep anti-(mouse IgG) antibody (Amersham International, code RPN 1001) or donkey anti-(rabbit IgG) antibody (Amersham International, code RPN 1004), diluted 1:100 as above with gelatin/Tris–saline containing pig IgG. Incubation with second antibody was for 1 h at 20 °C. After a washing, streptavidin–biotinylated horseradish peroxidase complex (Amersham International, code RPN 1051) diluted 1:400 in gelatin/Tris–saline was applied for 1 h. The peroxidase substrate was prepared by dissolving 3-amino-9-ethylcarbazole (Sigma, code A5754) in a small volume of *NN*-dimethylformamide, and diluting into 50 mM-sodium acetate buffer, pH 5.0, to give a final

concentration of 0.2 mg/ml. After filtration and addition of H_2O_2 to 0.03%, samples were incubated at 20 °C for an appropriate time for adequate staining. Between all steps the samples, in racks, were washed by immersion with gentle stirring in 400 ml of Tris–saline. Samples were mounted in 90% (v/v) glycerol in 150 mM-NaCl/50 mM-sodium phosphate buffer, pH 8.6.

Double labelling

The mouse monoclonal antibody, 40D, had been raised against rat MHC class II antigens by Dr. Pierres (Pierres *et al.*, 1981) and is known to cross-react with I-E molecules from pigs (Lunney *et al.*, 1983). The antibody was donated to us by Dr. M. D. Pescovitz, National Institutes of Health, Bethesda, MD, U.S.A. For double labelling RP 109 antibody was biotinylated (Pescovitz *et al.*, 1985) and detected with Texas Red–streptavidin (Amersham International, code RPN 1233); 40D antibody was detected as described for GK7C2 antibody. Controls were performed by omitting one or other or both first antibodies and replacing with pre-immune serum from the same species.

Other methods

The assay of endopeptidase-24.11 with ^{125}I-labelled insulin B-chain, protein and carbohydrate determinations and SDS/polacrylamide-gel electrophoresis were performed as previously described (Fulcher & Kenny, 1983; Fulcher *et al.*, 1983).

RESULTS

Isolation of endopeptidase-24.11 from lymph nodes

The enzyme was purified from mesenteric lymph nodes by the same steps that were effective in isolating the enzyme from kidney (Gee *et al.*, 1983). A microsomal membrane pellet was treated with Triton X-100 and the solubilized extract chromatographed on DEAE-cellulose before binding of the antigen to a GK7C2 monoclonal antibody column. It was eluted at pH 10.6 (Table 1). The purified protein was homogeneous on SDS/polyacrylamide-gel electrophoresis, where it migrated as a 91000-M_r polypeptide (Fig. 1), intermediate in size between preparations of the endopeptidase from kidney and intestine. The last two are glycoproteins of subunit M_r 87000 and 95000, and the size difference has been attributed to variations in the pattern of glycosylation (Fulcher *et al.*, 1983). One sample of the enzyme from lymph nodes had the following carbohydrate composition (μg/mg of protein): fucose, < 0.9; mannose, 18.0; galactose, 16.9; *N*-acetylgalactosamine, ~ 2.0; *N*-acetylglucosamine, 32.6; *N*-acetylneuraminate, ~ 3.0; glucose, 16.9. The pattern, with respect to the lower value for fucose and higher content of *N*-acetylneuraminate, more closely resembles the kidney enzyme than that from intestine. The specific activity of the enzyme (152

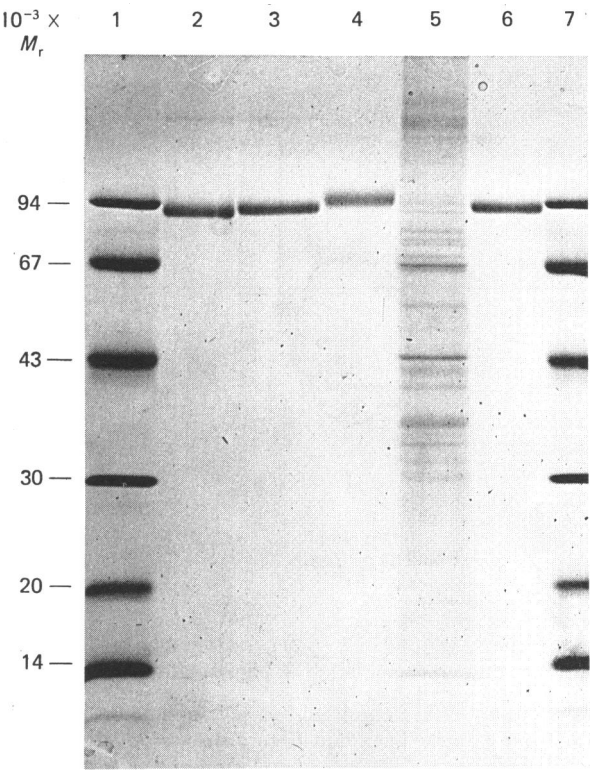

Fig. 1. SDS/polyacrylamide-gel electrophoresis of endopeptidase-24.11 purified from lymph nodes, kidney and intestine

See the Experimental section for details. The gradient gel comprised 7–17% (w/v) polyacrylamide. Track 1, marker proteins (phosphorylase *b*, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, α-lactalbumin); track 2, kidney endopeptidase-24.11 (5 μg); track 3, lymph-node endopeptidase-24.11 (5 μg); track 4, intestinal endopeptidase-24.11 (5 μg); track 5, lymph-node microsomal membranes (50 μg); track 6, as track 3; track 7, as track 1.

Table 1. Purification of endopeptidase-24.11 from mesenteric lymph nodes

Endopeptidase-24.11 was purified from 200 g of pig mesenteric lymph nodes, as described in the Experimental section. Activity was assayed by using ^{125}I-labelled insulin B-chain as substrate.

Fraction	Protein (mg)	Endopeptidase activity: Total activity (munits)	Endopeptidase activity: Specific activity (munits/mg of protein)	Endopeptidase activity: Yield (%)	Endopeptidase activity: Purification factor
Homogenate	36300	5130	0.14	100	1
Microsomal pellet	880	928	1.05	18	7.5
Triton X-100-solubilized fraction	440	808	1.8	16	13
DEAE-cellulose chromatography	280	730	2.6	14	18
Immunoadsorbent chromatography	1.5	228	152	4.4	1090

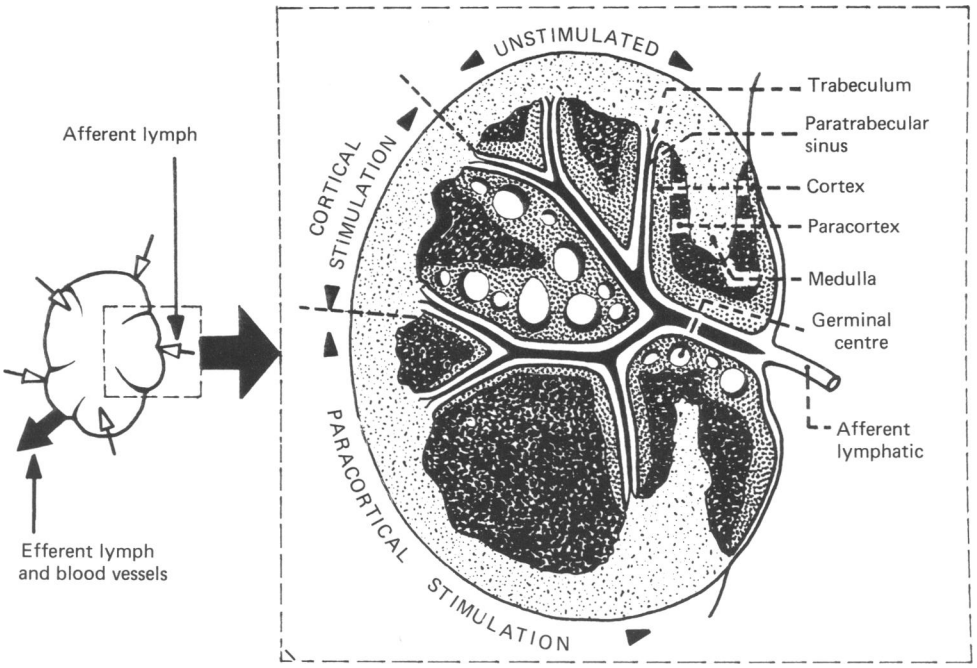

Fig. 2. Schematic representation of pig lymph-node structures

Large pig nodes are composed of several smaller nodelets, each supplied with an afferent lymphatic vessel, as shown on the left of this Figure. The main Figure shows an enlargement of a single nodelet, showing, in three segments, the effects of zero stimulation, cortical (B-cell) stimulation and paracortical (T-cell) stimulation. This figure has been redrawn after Binns (1982).

munits/mg of protein) was comparable with that of kidney (Fulcher & Kenny, 1983), intestine (Fulcher *et al.*, 1983) and brain (Relton *et al.*, 1983).

Regional distribution of endopeptidase-24.11 in lymph nodes

The interpretation of the micrographs of lymph nodes requires some understanding of their rather peculiar functional anatomy in the pig, the organization of which has been reviewed by Binns (1982), and is shown in Fig. 2. Essentially, pig lymph nodes are structurally inverted, possessing peripheral 'medullary' tissue and central 'cortical' tissue. In addition, large nodes are composed of several autonomous nodelets, each supplied with an afferent lymphatic duct, which carries lymph into the centre of the nodelet via the trabeculae. The lymph then passes through the central cortex, containing follicles and germinal centres, and on to the T-lymphocyte-rich paracortex, which is characterized by large numbers of 'high-endothelial' blood vessels. From here the lymph passes through the peripheral medulla, which is largely composed of cords, sinuses and blood vessels but relatively depleted of lymphocytes, before emerging into the efferent lymphatic vessel. Lymphocyte traffic differs in the pig from that in other species in that very few lymphocytes leave the node by the efferent lymph, but rather migrate directly into the blood via the 'high-endothelial' vessels.

The variation in endopeptidase content from node to node noted in the i.r.m.a. survey (Gee *et al.*, 1985) was confirmed by immuno-peroxidase cytochemistry (Fig. 3). Although small differences were seen between matched nodes from the same animal (cf. Fig. 3*a* panels 3 and 6), the major variation appeared between lymphocyte-rich (e.g. Fig. 3*a* panel 6) and lymphocyte-depleted nodes (Fig. 3*a* panel 2). Where very intense staining was seen the tissue contained few lymphocytes, being essentially composed of cords and sinuses, typical of medullary tissue (shown magnified in Fig. 3*b*). Conversely, in lymphocyte-rich areas, staining was less intense or even absent (Fig. 3*c*). It appears therefore that the total amount of medullary tissue present in any node is a major factor in determining endopeptidase-24.11 content of that node.

The pattern of immuno-staining was reticular in all regions of the node, and this was particularly pronounced when the animal was perfusion-fixed, as shown in the medulla (Fig. 4*a*) and paracortex (Fig. 4*b*). In over 60 separate lymph nodes examined, endopeptidase immunoreactivity was invariably present in the medulla, where it was localized to the medullary cords, particularly in cells that completely encircled sinuses and blood vessels (Fig. 4*c*). A comparable appearance was observed in the paracortex, where positively stained cells encircled 'high-endothelial' vessels (Fig. 4*d*). Staining was absent from endothelial cells and the lumina of sinuses.

Further details of representative regions can be seen in peroxidase-stained sections (Fig. 5). In Fig. 5(*a*) two follicles are seen in which the staining is concentrated at their centres, but with a zone of intense staining surrounding them. The trabeculae and lining of vessels and sinuses were not stained. Another view of the medulla (Fig. 5*b*) shows dense staining around vessels and sinuses. Within the paracortex, the intensity of staining varied widely (Figs. 5*c* and 5*d*). Staining was often more intense in the area immediately adjacent to trabeculae, but this reticular staining was usually absent within large aggregations of lymphocytes (Figs. 5*c* and 5*d*). Staining of lymphocytes was never observed in sections, or in peripheral blood, or in lectin-stimulated lymphocyte cultures. The last were analysed by using GK7C2 as the primary antibody and a fluorescein isothiocyanate-conjugated second antibody in a fluorescence-activated cell sorter (by Dr. R. M. Binns, A.F.R.C.

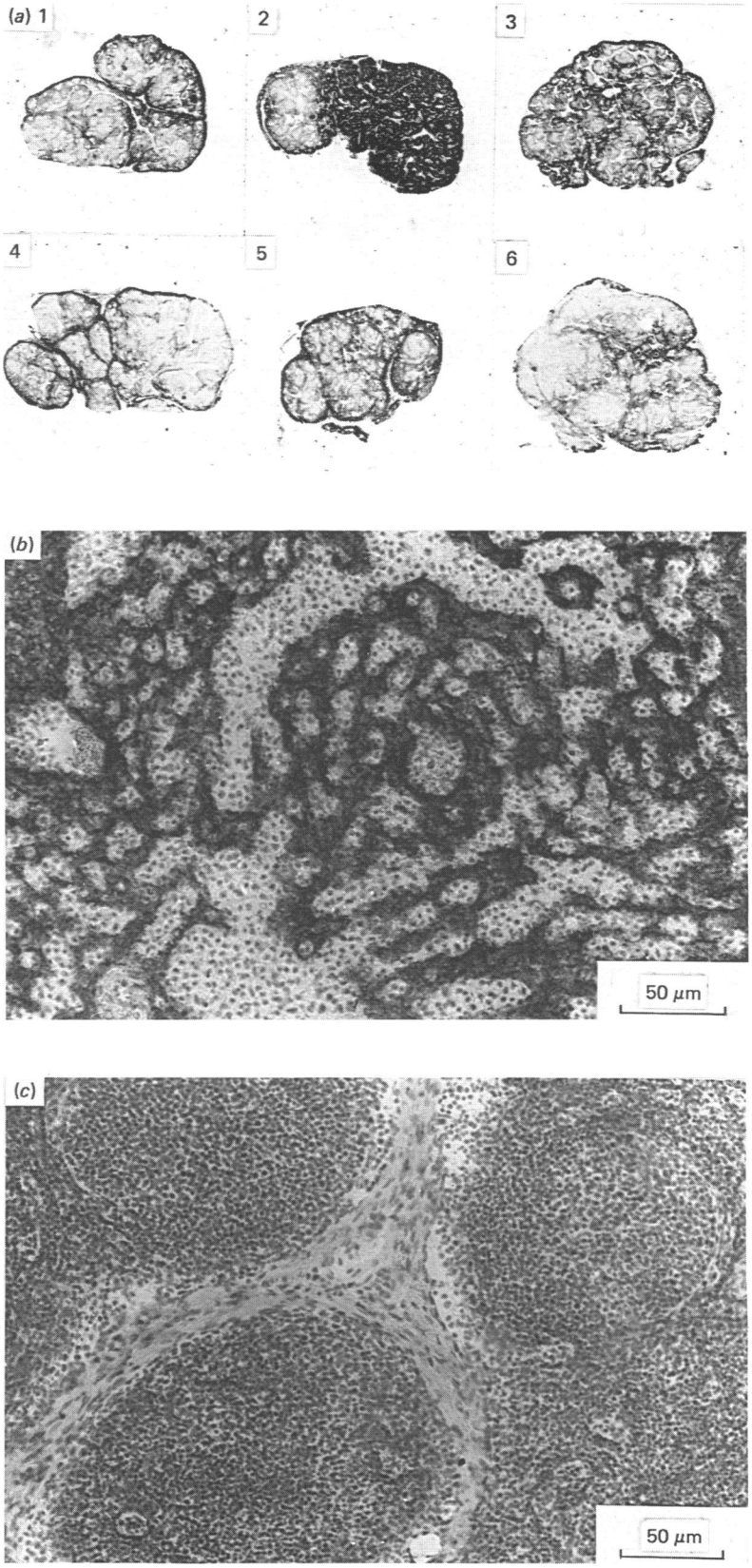

Fig. 3. Immuno-peroxidase staining for endopeptidase-24.11 of pig lymph nodes to illustrate the variation in staining intensity

(*a*) Six lymph nodes from same animal (fresh blocks, acetone-fixed and not counterstained): 1, mandibular (L); 2, prescapular (L); 3, parotid (L); 4, mandibular (R); 5, suprainguinal (R); 6, parotid (R). (*b*) Higher magnification of intensely stained region of (*a*) panel 2 (counterstained). (*c*) Higher magnification of weakly stained region of (*a*) panel 2 (counterstained). The primary antibody was GK7C2.

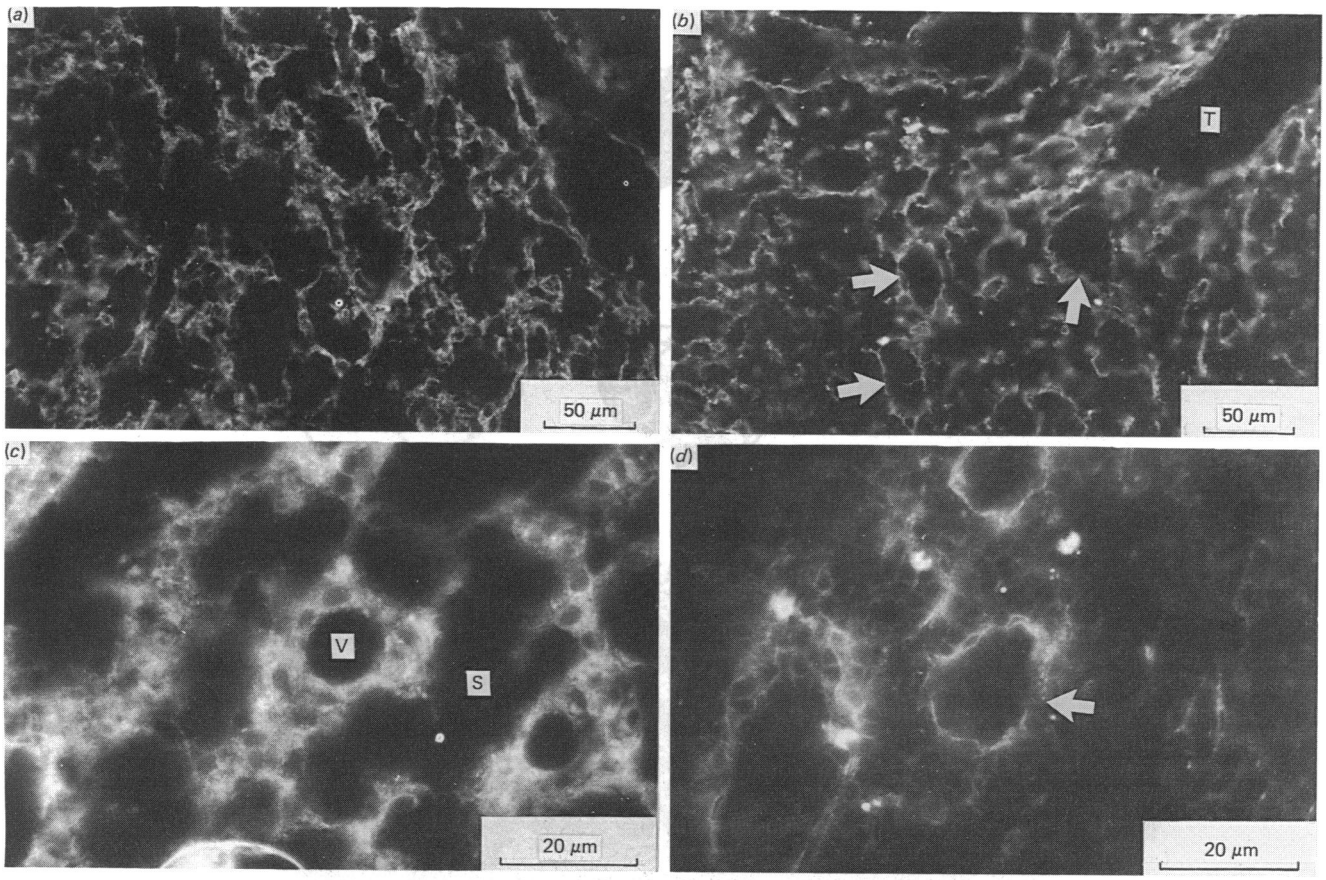

Fig. 4. Immunofluorescent staining for endopeptidase-24.11 in lymph nodes

The nodes had been perfusion-fixed and sections were stained with RP109 as the primary antibody (see the Experimental section for details). (*a*) and (*c*) Medulla viewed at low and high magnification respectively. (*b*) and (*d*) Paracortex at low and high magnification respectively. The reticular nature of the staining is well seen in both regions of the nodelet. V, vessel surrounded by intense immunostaining; S, sinus; T, trabeculum, which is unstained but is adjacent to areas of intense staining. Arrows indicate 'high-endothelial' vessels, also surrounded by intense reticular staining.

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Primary cell cultures from lymph nodes

Enzymic treatment of the nodes was essential: without it the yield of intact cells was low and survival in culture was poor. After enzymic dissociation, a heterogeneous population of cells was released, of which only approx. 0.2–1.0% stained positively for endopeptidase (1000 cells examined in each of three experiments). The recognition of positive cells was sometimes difficult owing to intense autofluorescence in some cells, as well as to positively staining membrane fragments adherent to otherwise non-staining cells. However, when intact, the positively staining cells were round in appearance (Figs. 6*a* and 6*b*), with a diameter about twice that of the numerous small lymphocytes. The cytoplasm of these cells was pale, granular and relatively abundant. The plasma membrane was strongly fluorescent, especially at the circumference, where the presence of granules added to the intensity.

After culturing overnight, approx. 5–10% of the total cell population became adherent to glass or plastic, in that they were not dislodged by gentle washing with a Pasteur pipette. The non-adherent fraction contained very few positive cells (< 0.01%), but a substantial proportion (5–25%) of the adherent cells now stained positively for endopeptidase-24.11 and these positive cells comprised two morphologically distinct types, present in approximately equal numbers. The first group of cells were of a flat fibroblast-like morphology, rich in intracellular vesicles, when examined by phase-contrast microscopy. Staining was always observed in the plasma membrane and often in the intracellular vesicles, which were notably free of intense autofluorescence (Figs. 6*c*, 6*d* and 6*e*). The flatness of the cells made it difficult to resolve the plane of the vesicles from the surface membrane. However, the fluorescent granules in Fig. 6(*d*) could often be identified with vesicles revealed by phase-contrast microscopy as in Fig. 6(*c*). This type of staining persisted during the first 2 days of culture, without change in either the frequency of positive cells or the intensity of staining. At this stage of culture, lymphocytes appeared to be firmly attached to the surfaces of many of these immuno-positive cells (Figs. 6*f*, 6*g* and 6*h*). After 2 days the number of positive cells decreased rapidly while the number of non-staining fibroblasts increased, becoming confluent within 6–10 days. These fibroblasts differed in appearance from the endopeptidase-positive cells in that they contained fewer vesicles, possessed a more dense cytoplasm and a more strongly defined nucleus containing two nucleoli. Staining for endopeptidase was not detectable in any cells after 4 days of culture. At no time did endopeptidase-positive

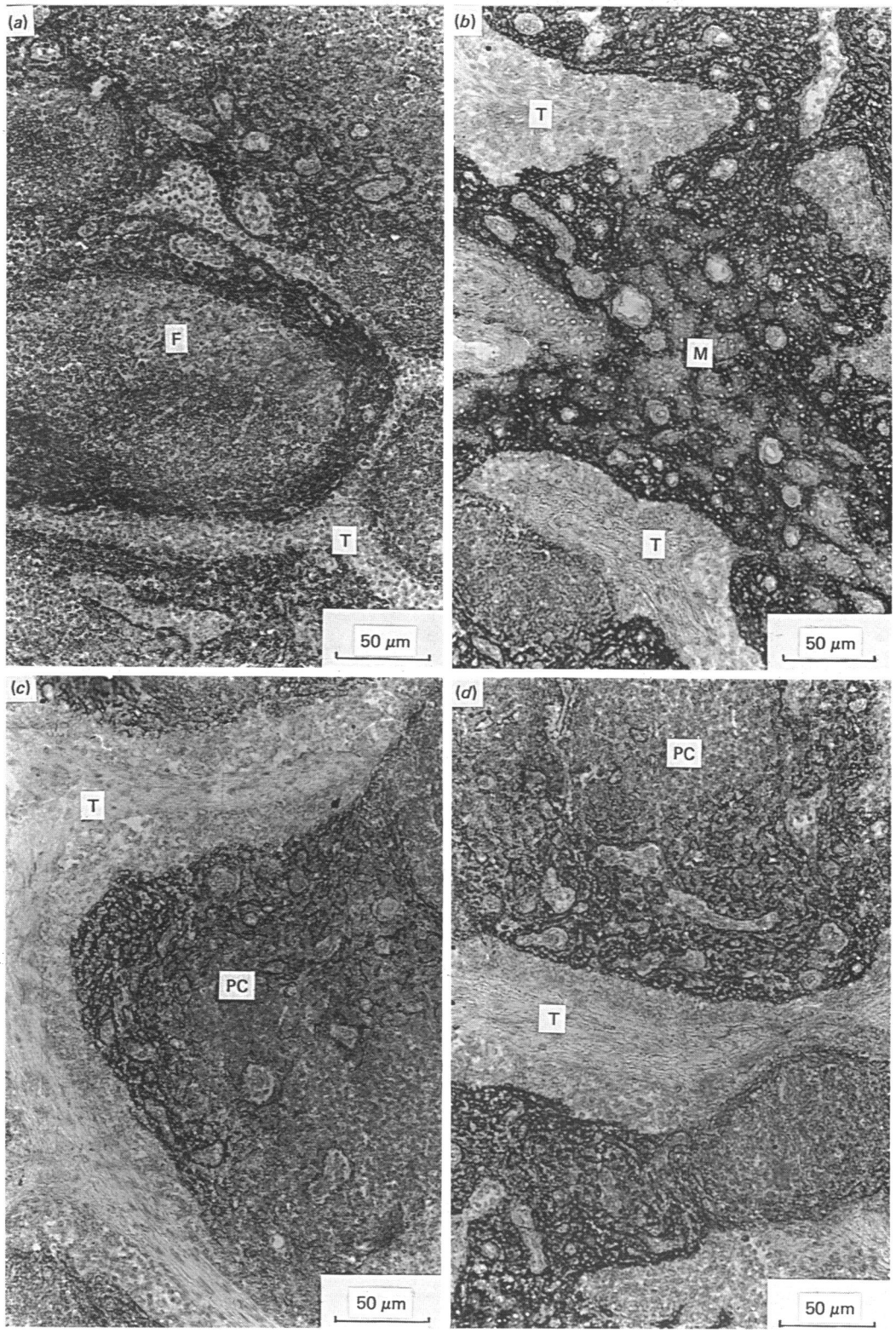

Fig. 5. Immuno-peroxidase staining for endopeptidase-24.11 in a lymph nodelet

See the Experimental section for details. The sections were from fresh blocks, paraformaldehyde-fixed. The primary antibody was GK7C2 and sections were counterstained. (*a*) Cortex showing two follicles (the larger marked F) with centres showing immunostaining, but with more intense staining peripheral to each follicle and adjacent to the trabeculae (T). (*b*) Medullary region, the typical appearance of medulla is indicated (M). The trabeculae (T) are unstained. (*c*) and (*d*) Paracortical region. The typical appearance of paracortex is indicated (PC). The most intense staining is adjacent to the trabeculae (T).

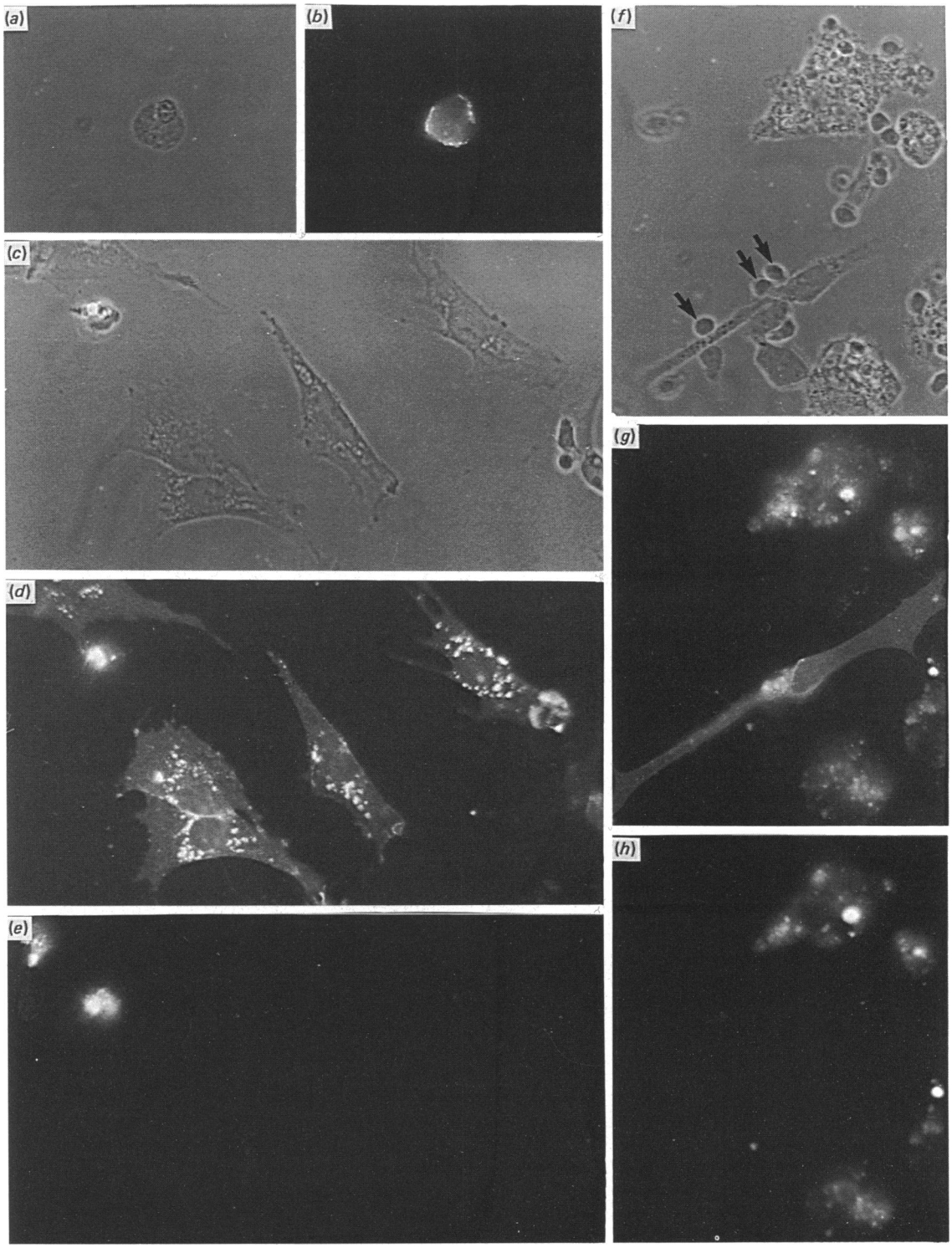

Fig. 6. Immunofluorescent staining for endopeptidase-24.11 in cells isolated from a lymph node

(*a*) and (*b*) Phase-contrast and fluorescence microscopy of a cell examined immediately after isolation. The fluorescence is present on the membrane surface, intensified around the circumference by fluorescent granules. (The primary antibody was RP109.) (*c*), (*d*) and (*e*) Phase-contrast, immunofluorescence and autofluorescence microscopy respectively of cells cultured for 2 days. These adherent cells now have the appearance of fibroblasts, the cytoplasm of which, viewed by phase contrast (*c*), is rich in vesicles. The membrane shows diffuse fluorescence together with brightly fluorescent vesicles, which are not autofluorescent (*c* and *d*). The primary antibody was RP109. (*f*), (*g*) and (*h*) Phase-contrast, immunofluorescence and autofluorescence microscopy respectively of another culture at 2 days. The very long fibroblastic cell can be seen to have attached lymphocytes (arrows in *f*). This cell also shows diffuse surface immunofluorescence as well as more intense staining of intracellular vesicles (the primary antibody was GK7C2). The lymphocytes have not stained. In addition, there are large round cells resembling macrophages, the granules of which are brightly autofluorescent (*h*).

cells stain positively for Ia antigen, with the 40D antibody, in a double staining protocol.

The second type of cell exhibiting positive immunoreactivity was also round in shape but was characterized by an abundance of autofluorescent granules, typical of macrophages. However, some of the granules also exhibited positive immunostaining for endopeptidase-24.11. Since the plasma membranes of these cells were negative and since tissue sections never revealed macrophage-like cells in the 'empty' regions of nodes, it is likely that these cells had acquired their immuno-positive inclusions by phagocytosis and could not be regarded as cells expressing this antigen in their membrane.

DISCUSSION

Endopeptidase-24.11 has been localized in lymph nodes by two antibodies. The first, GK7C2 antibody, was a monoclonal antibody previously shown to recognize the enzyme in various tissues by 'Western' blotting, immunoprecipitation, i.r.m.a., immunoaffinity chromatography and immunofluorescence microscopy (Gee *et al.*, 1983, 1985). The second, RP109 antibody, was a specific polyclonal antiserum raised in rabbits, and which had been further refined by elution from a column to which purified antigen had been bound. Both antibodies gave similar immunocytochemical appearances in this study on lymph nodes, and we may conclude that the antigen recognized was endopeptidase-24.11. The intensity of staining of lymph nodes was consistent with our earlier finding that pig lymph nodes were an abundant source of the enzyme. Its purification by immunoaffinity chromatography revealed an enrichment of 1000-fold (cf. 150–180-fold for kidney, 1200-fold for intestine; Fulcher & Kenny, 1983; Fulcher *et al.*, 1983), and the purified enzyme had the same specific activity as that from other organs of the pig. Carbohydrate analysis indicated differences from that for the kidney and intestinal preparations, and this may explain the slightly different subunit M_r for the preparation from lymph node.

The variability of endopeptidase-24.11 content noted in the i.r.m.a. study (Gee *et al.*, 1985) was also apparent in the variation of intensity of immuno-peroxidase staining from node to node. The most intense staining corresponded to regions of the node containing few lymphocytes. Thus the medulla showed consistent staining, although the depth of this staining beneath the capsule varied considerably. Nevertheless, endopeptidase-24.11 was also demonstrable in the other main regions of the nodelet, the cortex and paracortex, including the centres of the follices. No immunoreactivity was seen within vessels or sinuses or in the connective tissue and other components of the trabeculae. The pattern of staining when viewed at sufficient magnification was similar in all regions, namely a reticular appearance enmeshing unstained cells, frequently concentrated around vessels, sinuses and follicles, and often intense in areas adjacent to the trabeculae.

It was clear from the examination of tissue sections, as well as from the failure of lymphocytes to stain *in vitro*, that the antigen was expressed by cells other than lymphocytes. The culture of cells obtained by mechanical and enzymic disruption of lymph nodes provided clues to the nature of the immuno-positive cells. They were characterized as follows. (1) In suspension they were round cells with intense staining of the plasma membrane. (2) They quickly became adherent to plastic or glass and remained so during culture over several days. (3) They were Ia-negative. (4) They were fibroblastic in appearance, containing many intracellular vesicles that were immuno-positive, as was the surface membrane. (5) In the early period of culture, they bound many lymphocytes. (6) Immuno-positive cells were not visible after 4 days in culture, being replaced at confluence by cells differing slightly in morphology from the immuno-positive cells. Whether this change represented overgrowth by a different cell type or de-differentiation during culture is not yet clear. This description of the immuno-positive cells best fits that of the reticular cells (Stuart & Davidson, 1971; Haston, 1979). *In vivo*, these cells are firmly attached to the network of reticulin, which they are believed to lay down and which is essential for stabilizing the anatomical structure of lymph nodes. Since the reticular network, and hence the reticular cells, are present throughout the node, it was surprising to find that the distribution of endopeptidase-24.11 was both non-uniform and variable in amount. The observation suggests that the expression of this antigen denotes a differentiated form of the reticular cell with a functional requirement for a surface endopeptidase.

The observation that vesicles, apparently intracellular, were strongly immunofluorescent was unexpected for an antigen that, in other tissues, is confined to the plasma membrane. The intensity of the fluorescence seems to be inconsistent with the processes of bulk pinocytosis and membrane recycling, but rather it suggests selective enrichment of the antigen during formation of the vesicles. It is possible that the method for isolating the cells, with the use of proteinases, may have led to an artifactual redistribution of the enzyme. However, two arguments make this interpretation unlikely. First, a comparable treatment used to isolate chondrocytes from cartilage yielded cells exhibiting only surface staining for endopeptidase-24.11 (Gee *et al.*, 1985). Secondly, the intense vesicular immunofluorescence was not a transient phenomenon detected only in the first few hours after isolation of the cells. It persisted for at least 2 days, during which the distribution of the vesicles changed from being peripheral to one that was predominantly perinuclear.

Dendritic cells, which are involved in antigen presentation, have crucially different characteristics from those cells that we have observed to stain positively for endopeptidase-24.11 (Steinman *et al.*, 1979; Szakal *et al.*, 1985; Schnizlein *et al.*, 1985). In the present context, the important points are their inability to remain adherent to surfaces during culture and their invariable expression of Ia antigen on their surfaces. We observed many Ia-positive cells in the suspension immediately on isolation, but none of the fibroblastic cells adherent on the second day of culture was Ia-positive. It is clear that endopeptidase-24.11 is not expressed by dendritic cells, and this observation may suggest that the enzyme is not involved in antigen processing.

We have postulated a role for endopeptidase-24.11 in the nervous system in the metabolism of neuropeptides (Matsas *et al.*, 1983), and it is tempting to suggest that it might serve a similar function in the immune system. It is now apparent that there is a close relationship between the immune and neuro-endocrinological systems. An early observation was that lymphoid cells produce corticotropin-like and endorphin-like substances (Smith

& Blalock, 1981; Smith *et al.*, 1982). Lymphocytes express the pro-opiomelanocortin gene and exhibit an ability to respond to corticotropin-releasing hormone; in addition, both endorphins and enkephalins have been shown to modulate the immune response (Smith *et al.*, 1985). Lymphocytes also have receptors for neurohypophysial peptides, and oxytocin and [arginine]vasopressin can replace interleukin-2 as a factor in the production of γ-interferon in mouse spleen-cell cultures (Johnson & Torres, 1985). Lymphocytes have receptors for substance P, somatotropin and vasoactive intestinal polypeptide, and these three peptides modulate the immune responses of lymphocytes, the last being mediated by cyclic AMP (Payan & Goetzl, 1985; O'Dorisio *et al.*, 1985). Peptides together with lymphokines and thymosins thus constitute a group of substances, that have been referred to as 'immunotransmitters' (Hall *et al.*, 1985). We suggest that endopeptidase-24.11 in lymph nodes may be involved in the termination of some of these 'immunotransmitter' signals, and among these the most likely substrates are peptides, since the enzyme seems to have little activity against native proteins (Kerr & Kenny, 1974). However, the high concentration of the endopeptidase in the medulla, where lymphocytes were few, and the variable content of the enzyme from node to node, are additional points awaiting functional explanations.

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